

Role of Cytochrome P450s (CYP) in Isoniazid Bioactivation

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Submitted to the Graduate Faculty of
School of Pharmacy in partial fulfillment
of the requirements for the degree of
Master of Science

University of Pittsburgh

2019

UNIVERSITY OF PITTSBURGH
SCHOOL OF PHARMACY

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Tuberculosis (TB) is an infectious disease caused by bacteria *Mycobacterium Tuberculosis*. With a huge population infected worldwide and millions of new cases reported on an annual basis, TB is a serious threat to global health. TB chemotherapy is a crucial strategy and isoniazid (INH) is one of the most potent anti-TB drugs that is heavily used in both TB chemoprophylaxis and treatment.

One of the biggest concerns in INH therapy is INH-induced liver injury (I-ILI). Marked by persistent elevation of serum alanine aminotransferase (Alanine Aminotransferase and Aspartate aminotransferase), usually ten times above upper limit of normal (ULN), it is characterized as hepatocellular injury of a phenotype happened in 0.5-1.0% of patients on INH treatment. These patients are facing a high risk of fatal liver failure. INH associated hepatotoxicity was identified as early as its introduction to the clinical use but the mechanism remains elusive. As numerous studies propose myriads of potential mechanisms, immune-mediated response was considered important contributing factor towards INH associated hepatotoxicity recently.

Since small molecules like INH and its metabolites are less likely to trigger the immune response, complexes of INH or its metabolites with endogenous macromolecules are hypothesized as antigens in this case. The study targeted on INH-human serum albumins (HSAs) adducts showed that this complex did get recognized by the immune system, but auto-antibodies for this antigen was undetectable even with highly sensitive radioimmunoassay. Subsequently, attention

was switched to the CYP-bioactivated INH-CYP adducts and its association with I-ILI. In this case, auto-antibodies of these adducts were detectable, but the products of CYP-mediated INH bioactivation along with isoforms of CYP involved remains unclear. To further address this question, we developed in vitro incubation systems to identify the existence of CYP-mediated INH bioactivation products and the CYP isoforms responsible for the bioactivation.

To detect bioactivation products, we introduced N- α -acetyl-L-lysine (NAL) as a trapping agent to stabilize them and make them detectable as INH-NAL adducts. Results from incubation shown INH went through CYP-independent auto-oxidation, and no bioactivation products were observed in three individual CYP isoforms and human liver microsomes we evaluated. Thus, our results do not support the hypothesis of I-ILI that is mediated by the immune response from CYP-bioactivated INH-CYP adducts.

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Preface

I would like to thank Dr. Xiaochao Ma for his patience, support, and guidance during my study of master's degree, I am appreciative to work with him.

I am very grateful for my lab members, Lucy Jie Lu, Pengcheng Wang Ph.D., Junjie Zhu Ph.D., Amina I. Shehu for their support and advises on both my research and life decisions.

My appreciation also goes to Ms. Lori M Altenbaugh, Dr. Maggie Folan and all the staff, faculty members as well as students in the School of Pharmacy, University of Pittsburgh.

Introduction

1.1 Overview of Tuberculosis (TB)

Tuberculosis (TB) is one of the biggest global health issues due to the population it infects worldwide. One quarter to one-third of the global population has been infected with TB. In 2016, there are 6.3 million new TB cases and about 1.7 million cases of death due to TB. On top of that, 95% of the death cases happened in developing countries. ¹

Cause of TB was firstly revealed by Dr. Robert Koch announcing his discovery of *Mycobacterium Tuberculosis* as the pathogen of TB. ² This is considered as the very first step to the cure of TB, a disease so deadly that it had killed one-seventh of the population lived in America and Europe, by the time of this discovery.

1.2 Overview of Isoniazid (INH)

Effective chemotherapy for TB treatment started in 1944 and many compounds had been developed ever since. ³ Among these drugs, Isoniazid (INH) is one of the most potent, first-line anti-Tuberculosis (Anti-TB) drugs available in clinical practice.

Initially synthesized in 1912, INH was never considered as an anti-TB drug by its entrance. ⁴ Later at the development of TB chemotherapy, INH appeared as an intermediate of isonicotinaldehyde thiosemicarbazone synthesis, and therefore, it was tested for its anti-TB effect due to its structural similarity with isonicotinaldehyde thiosemicarbazone. Unexpectedly, this

intermediate outweighed all other candidates with its excellent activity and moved on to further investigations under the name of Rimifon.^{4 5} With high potency and good safety profile, INH became the wonder drug of TB chemotherapy and finally marketed in 1952. Combination of streptomycin (MS), para-aminosalicylic acid (PAS) and INH covered almost all the resistant strains of *Mycobacterium tuberculosis* (MTB) and majorly decreased mortality of TB in the 1960s.

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Nowadays, INH is the first choice for TB chemoprophylaxis; it is also recommended for the treatment of latent TB and can be combined with other anti-TB drugs for active TB. INH treatment is crucial and life-saving in certain cases. For instance, liver transplant recipients, especially those who have latent TB, can face as high as 20 to 74-fold risk of active TB infection than the general population due to immunosuppressive therapy. With INH chemoprophylaxis, this risk is significantly reduced and directly leads to an improved survival rate of patients.^{6 7} Also, for latent TB treatment on liver transplant candidates, INH prevents them from progressing into active TB and reduced post-transplantation mortality from TB infection.⁷ Thus, INH is one of the key factors that heavily impacts the management of TB in various stages.

1.3 INH-induced liver injury (I-ILI)

1.3.1 Overview of I-ILI

Apart from its potency as an antibiotic in both bacteriostatic and bactericidal manners, adverse effects of INH (including epigastric distress, neurotoxicity, hepatotoxicity, and allergic reactions) were reported right after its clinical application. For instance, one of its earliest

hepatotoxic events was revealed in 1952, the same year as it was marketed. In this case, a 46-year-old male patient was hospitalized with toxic hepatitis after one month of treatment with INH.⁸ Same as shown in this case, most of INH's hepatotoxic events resemble acute fetal hepatitis. Guidelines⁹ were built afterward to monitor liver functions and protect patients. Following these guidelines and having patients on regular hepatotoxic surveillance decreased the previously underestimated incident rate, but the cause of them was poorly understood.

Clinically, as many as 80% to 90% of the patients on/post INH treatments show no sign of liver injury. 10% to 20% of patients had a transient elevation of aminotransferases, the classic biomarker for general liver injury. For these patients, their readings of aminotransferase will fall back to normal range within a few weeks. Thus, they were still considered safe to continue INH treatment. Still, there is about 1% of patients on INH treatment, unluckily, progress into the stage of fatal hepatitis.¹⁰

However, numbers here are just a smaller portion of the reality. I-ILI ranks second on the list of under-reporting drugs that cause hepatotoxicity.¹¹ In this study, as many as 60 cases were selected as the population of I-ILI as they met the American Thoracic Society (ATS) guideline of inclusion⁹. Despite 13 (22%) out of these 60 patients end up with some of the worst outcomes, death or liver transplantation, only one case was reported to the Centers of Disease Control, as it supposed to.¹² With this overwhelmingly underreported situation and the already-enormous population that undergo INH treatment, one can only imagine how many of us are under the threat of I-ILI. Without a doubt, TB is now the biggest killer among infectious diseases.

Patients in this rare case often hospitalized with lower abdomen pain, nausea, rashes, fever or loss of appetite. Blood tests of them show a long-term elevation of aminotransferase and a high

level of total bilirubin. Liver biopsy indicates hepatocellular injury with massive necrosis and inflammation.¹³

This fatal hepatitis from isoniazid-induced liver injury (I-ILI) has high mortality. Once I-ILI progresses into this stage, there are few options available for these patients. One of which is liver transplantation. Regardless of the riskiness of the liver transplant operation, a relapse of I-ILI might still occur after re-challenging of INH for TB treatment.

1.3.2 Potential mechanisms of I-ILI

Numerous potential mechanisms of I-ILI were suggested by scientists within the past six decades, while the true story behind these cases remains unknown. These mechanisms include the formation of reactive/toxic metabolites of INH, disturbance of endobiotic homeostasis and the immune response targeted INH/metabolites-protein adducts. Along each mechanism, variants from both patients (age, gender, race, genetic polymorphism, etc.) and drugs (reactive metabolites, interaction with endobiotic/proteins, etc.) are considered as factors potentiate the I-ILI.¹⁴

1.3.2.1 Formation of reactive metabolites (RMs)

One of the very first hypothesis researchers offered about I-ILI is from its metabolites.¹⁵ These metabolites of INH include Acetylhydrazine (AcHz), hydrazine (Hz) and acetylisoniazid (AcINH), etc. AcHz and AcINH were demonstrated as the cause of I-ILI in a rat model, but high-dose of INH itself failed to recapitulate the case^{10,16}. Then, in studies using mice, Hz is reported to be fully responsible for I-ILI with liver necrosis and steatosis, but the dose administered is way higher than the clinical observation.¹⁷ The same conclusion can also be obtained from a rabbit

study.¹⁸ Besides the controversial result of which metabolites causing the I-ILI, some of these studies also failed to phenocopy the case in clinical patients.^{13 19 20} Hz was also reported as a direct inhibitor of mitochondrial complex II, then leads to an energy crisis due to ATP production malfunction.²¹

Speaking of clinical patients, debates of which one causes I-ILI here is between INH and its metabolites. Either in rodents or human, *N*-acetyltransferase (NAT) is the one in charge of converting INH into its metabolites (AcHz, Hz and AcINH) directly and indirectly. The gene encoding NAT have high polymorphism. Those who categorized as rapid acetylators were thought to be potentiated to I-ILI at first^{22,23}, further studies suggest the other way around.²⁴⁻²⁷ Some observations kicked the rapid/slow acetylators' story out of the picture, indicating no significant difference between the two populations in the case of I-ILI.²⁸⁻³⁰

Overall, outcomes from the production of these RMs are majorly oxidative stress and mitochondrial toxicity.

1.3.2.2 Interactions with endobiotics

It has been reported that INH or its metabolite can bind to the endogenous molecules, including micro-molecules like pyridoxal (PL, vitamin B6) and NAD⁺^{31,32}.

In fact, peripheral neurotoxicity of INH is the outcome of INH-B6 (Pyridoxal phosphate, PLP) formation.³³ As early as 1952, a patient complained about his “burning feet” after using INH.¹⁷ Then the mechanism of this peripheral neuropathy was revealed. In vivo, INH can form covalent binding with PLP, which is a crucial co-factor for multiple enzymes.^{34,35} Once the bind forms, a depletion of PLP can be observed, along with the dysfunction of enzymes which need PLP as co-factor. Since many enzymes in the liver are also PLP-dependent, PLP depletion was also studied

in the case of I-ILI. However, people have not yet found any PLP-dependent enzyme that can dominantly initiate I-ILI.

Disturbance of endogenous homeostasis also happens to small molecules like bile acids and triglycerides, which may also contribute to the initiation and exacerbation of I-ILI.^{36,37}

1.3.2.3 Immune-mediated responses

One of the biggest problems from these two categories, formations of reactive metabolites and disturbance of endobiotic homeostasis, is the mismatch of the phenotype observed from patients. As rodents, including mice^{16 38 39 40}, rats^{19 13 41 42} and rabbit^{16 43 44 45}, are the major models investigated in studies above, one possibility is the species differences. Some might argue that mice are better of a model to resemble human, but neither hepatocellular necrosis nor elevation of serum aminotransferase level was captured, not to mention the request of dose relevance with clinical cases.¹⁰ Therefore, researchers start to search for answers from patients, directly. This time, they see hope from the auto/anti-drug antibodies existed in human samples.

Along with the suspicion of those well-known INH metabolites directly causing I-ILI, people also suspected their indirect manner out of a macro molecule-binding property and stimulating immune-mediated response.

A study has reported I-ILI patients, with either isoniazid-induced persistent elevation of serum glutamic oxaloacetic transaminase (SGOT, also known as Aspartate transaminase, AST) or isoniazid-induced overt hepatitis, shown 95% of positive rate (19 out of 20 patients) on *in vitro* lymphocyte transformation test (LTT) with stimulation by one or more of INH, isonicotinic acid (NA), and their conjugates with human serum albumin (HSA). For comparison, researchers recruited health subjects, as negative controls of INH treatment, as well as patients who never developed SGOT elevation, either in treating segment or had completed the treatment at the time,

as negative controls of I-ILI. ⁴⁶ LTT assay is one of the most common tools used in clinical diagnosis of drug hypersensitivity, which reflects direct interaction between T-cell receptor and drug/drug-protein adduct in vitro and a positive result refers to the pre-sensitized T-cells appearance in vivo. ⁴⁷ In this study, positive LTT readings from I-ILI patients indicates the existence of INH/INA/INH-HAS/INA-HAS recognition by the immune system, suggests immune response co-exists along with I-ILI. However, antibody detection from I-ILI patient serum by sensitive radioimmunoassay were negative. A point worth-of-mention was that this study excluded patients with hepatitis B. To patch up this part, a group of Hep B patients was also recruited as a control group. Their LTT results were negative, which ruled out the possibility of viral hepatitis as the initiator of immunologic recognition of INH, INA and their HSA conjugates. This study overall demonstrated the certain participation of immune-mediated response in I-ILI, but the absence of antibody suggested its character might be but a secondary phenomenon. ⁴⁶

Although the role of immune response in I-ILI was not clearly demonstrated from the study above, the idea of immune responses caught the attention of toxicologist. Later, their suspected hapten expanded from hydrolytic metabolites to other metabolites of INH, one of them is oxidized INH by CYP.

1.3.3 CYPs and isoniazid-induced liver injury (I-ILI)

1.3.3.1 Overview of CYPs

CYPs is a big family of heme-containing enzyme. They are widely distributed in different organs and play important roles in the metabolism of endobiotics and xenobiotics. ⁴⁸ In most cases, CYPs mediate oxidation at the end of a chain and using NADPH as a co-factor to transfer

electrons. CYPs have high abundance in human liver and therefore, serve as vital Phase I metabolism enzyme and increase the hydrophilicity of drug molecules. With the increase, if their hydrophilicity, drug metabolites have better solubility than their parent compound and are easier to eliminate from the system.^{49 50 51,52} Among all the CYP isoenzymes, 3A4, 1A2, 2C9, 2C19, 2D6 and 2E1 cover approximately 90% of the drugs have been used in medicine.⁴⁹

Also, CYP is known for its potentials to metabolize drugs into their reactive metabolites and trigger drug-induced liver injury (DILI). One of the most well-known cases is Acetaminophen (APAP)-induced liver injury mediated by CYP2E1. In this case, less than 10% of APAP was metabolized by CYP2E1 and form its RM, NAPQI. At the very beginning, GSH can serve as antioxidant thus liver has a certain tolerance. Once the production of NAPQI exceeded the GSH pool size, depletion of GSH with acute liver necrosis will be spotted.⁵³⁻⁵⁵

1.3.3.2 CYP-mediated INH bioactivation and I-ILI

CYP and its role in DILI have been established for decades, and INH bioactivation by CYP has been studied for a while.

CYP was previously reported participating in the metabolism of Hz and AcHz to their RMs, which may worsen the already existed I-ILI.^{56 57} While introducing a non-specific CYP inhibitor into the rat hepatocyte culturing, Hz-induced cytotoxic phenotype was abolished.⁵⁸ Moreover, co-treat INH with CYP inducer such as rifampicin can exacerbate the hepatotoxicity due to increased production of RMs.²⁰ This increased risk of I-ILI was reconfirmed in a clinical study, which showed a potentiate trend of I-ILI in patients on the combination of human Pregnane X receptor agonists/CYP inducers and INH.⁵⁹⁻⁶¹

CYP can also directly convert INH into its bioactivated form. Then, the bioactivated INH can form adducts with CYP and cause the immune response. One paper published in 2014⁶² shown

quite promising result from a study with patients. Anti-CYPs antibodies in their serum were detected. In this study, as high as 58% of (11 out of 19) patients with I-ILI have a positive reaction, while all patients who produced anti-CYP autoantibodies, including anti-CYP2E1, anti-CYP3A4, and anti-CYP2C9 developed INH-induced liver failure.⁶² All evidence above suggesting that these three CYPs are involved in INH bioactivation and leading to the formation of INH-CYP adducts and immune response.

1.4 Objectives of our study

As mentioned in the previous sections, CYP enzymes play a vital role in general drug metabolism and well-known for its potential as a mediator of drug-induced liver injury. In the case of INH, incubation with human liver microsome, which has a high abundance of CYP, can produce bioactivated INH. Therefore, they employed a trapping agent, NAL, to trap the reactive metabolite and looking into INH-NAL signal for bioactivation.¹⁶ Moreover, clinical studies indicate that bioactivated INH can form adducts with endogenous macro-molecules and stimulate the immune response. On top of that, patients with I-ILI has anti-CYP antibodies in their serum. As many studies throughout decades come to the same conclusion that CYP enzymes get involved the bioactivation of INH and immune responses the cause of I-ILI, there is still one piece of the puzzle missed in between, the exact CYP subtype(s) that participate.

In this study, our goal is to identify the individual CYP in charge of INH bioactivation, and the product(s) from CYP mediated INH bioactivation, if possible.

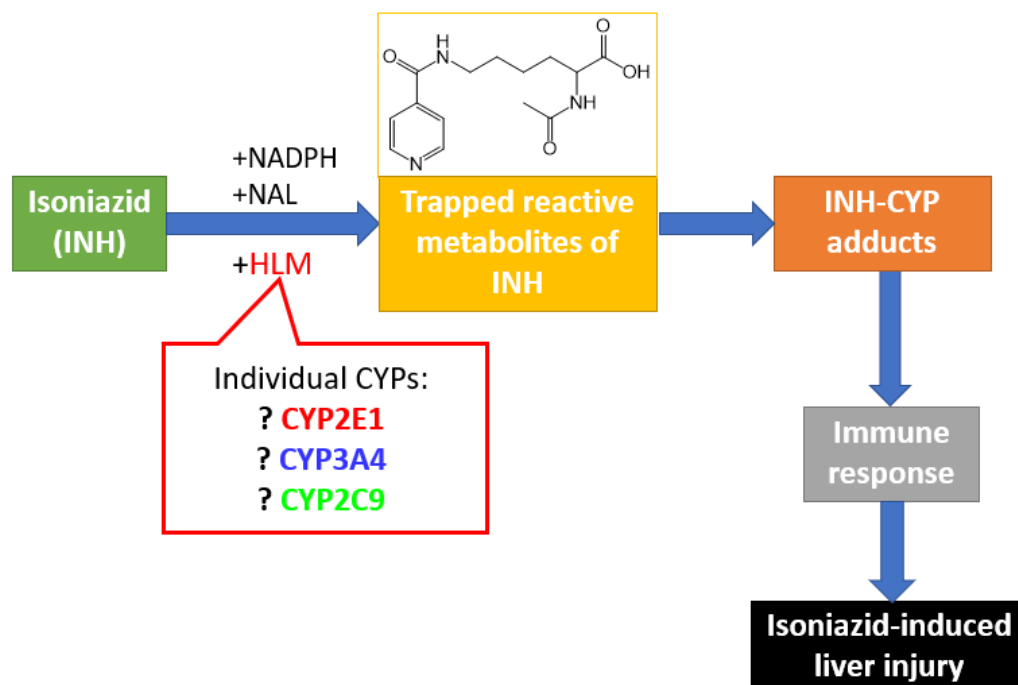


Figure 1 Study Objectives

We hypothesize that INH went through CYP-mediated bioactivation and formed reactive metabolites. Then, reactive metabolites can form adducts with CYPs and stimulate immune response, which finally lead to liver injury. Our study is to pinpoint which CYP isoform participate in INH bioactivation.

By identifying the individual CYP and clarifying the structure of INH bioactivation, we can further support the importance of immune response in I-ILI and validate the anti-CYP antibodies that reported earlier as a potential clinical biomarker to facilitate clinical diagnosis of I-ILI in its early stage.

2.0 MATERIAL AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Isoniazid (INH), L-glutathione (GSH), acetaminophen (APAP), Lopinavir (LPV), Diclofenac (DFC) and N-acetyl-l-lysine (NAL) were purchased from Sigma-Aldrich (St. Louis, MO). B-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Alfa Aesar (Tewksbury, MA). Recombinant human CYPs (EasyCYP Bactosomes), including rhCYP2E1, rhCYP3A4 and rhCYP2C9, human liver microsome (HLM) and NADPH regenerating system were purchased from XenoTech (Lenexa, KS).

Solvents, including water, methanol, and acetonitrile, for ultra-performance liquid chromatography-time-of-flight mass spectrometry (UPLC-TOF-MS), were LC-MS level; pH adjuster, formic acid (FA), was certified ASC level; solvents and pH adjuster for LC system were purchased from Thermo Fisher.

2.1.2 UPLC-TOF-MS system

UPLC system is Acquity UPLC I-class model from Waters, with Acquity UPLC BEH C18 column (2.1×100 mm, $1.7 \mu\text{m}$; Waters Corporation, Milford, MA).

Tof MS system is SYNAPT G2-Si ToF time-of-flight (ToF) model with Electrospray Ion-source (ESI) from Waters.

2.2 Methods

2.2.1 In vitro enzymatic incubation

For both validation and detection systems, substrates (including APAP, LPV, DFC, and INH) were incubated in 1 x PBS (pH 7.4) with reagents including cofactor of CYP (NADPH), trapping agents (GSH or NAL), and individual recombinant human CYP (2E1, 3A4, 2C9). Systems then were incubated at 37 °C.

Table 1 Components of CYP/HLM incubation systems

Enzyme/ microsome	Enzyme Conc.	substrate	Substrate Conc.	NADPH Conc.	Trapping agent Conc.	Incubation time
rhCYP2E1	100 nM	Acetaminophen	50 uM	1 mM	2.5 mM GSH	60 min
		Isoniazid	100 uM		2.5 mM NAL	90 min
rhCYP3A4	100 nM	Lopinavir	30 uM		2.5 mM GSH	30 min
		Isoniazid	100 uM		2.5 mM NAL	90 min
rhCYP2C9	100 nM	Diclofenac	30 uM		2.5 mM GSH	30 min
		Isoniazid	100 uM		2.5 mM NAL	90 min
HLM	1 mg/mL	Acetaminophen	50uM		2.5 mM GSH	60 min
		Isoniazid	100 uM		2.5 mM NAL	90 min

2.2.2 Incubation sample preparation for LC-MS detection

Terminating reaction and precipitating protein by adding 100 uL 50% of methanol in acetonitrile into the system. Mix well and then centrifuge at 15,000 rpm for 10 min under room temperature. Transfer supernatant into the sample vial.

2.2.3 LC-MS quantification of trapped products from CYP metabolism

2.2.3.1 Mobile phases and wash solutions

Mobile phase A and weak wash consisted of water with 0.1% formic acid. Mobile phase B and strong wash consisted acetonitrile with 0.1% formic acid. Seal wash consisted of water with 50% acetonitrile.

2.2.3.2 Chromatographic separation

Chromatographic separation achieved through gradient elution with an 8-min run. Elution gradient was shown as follow:

Table 2 Elution gradient of UPLC analysis

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	0.500	2.0	98.0	6
1	0.500	2.0	98.0	6
6	0.500	95.0	5.0	6
7	0.500	95.0	5.0	6
7.1	0.500	2.0	98.0	6
8	0.500	2.0	98.0	6

Temperature of the sample manager was maintained at 10 °C. Column temperature was maintained at 50 °C.

2.2.3.3 Mass spectrometry detection and data analysis

Detection was run under full scan mode, ranged 50-1000±0.05 Dalton (Da). All the detection was under resolution mode. Along with each injection, lock spray monitored the sensitivity of the detector to guarantee the reliability of response.

Data collection, as well as quantification, was done with Masslynx 4.1. Data analysis was done with GraphPad 8.0. All data presented in this thesis were in the template of mean±S.D. and statistically compared using one-way ANOVA.

3.0 Experimental Designs

3.1 Method validation for CYPs incubation system

To reconfirm that the supposed INH bioactivation is mediated by CYPs, here we employ probe substrates, whose CYP-mediated bioactivation had been clearly demonstrated, to validate our incubation system. These compounds go through specific CYP-mediated metabolism and form reactive metabolites (RMs). RMs can be trapped by glutathione (GSH) in the form of RM-GSH adducts. Then, RM-GSH adducts are detected by UPLC-MS system. To rule out the CYPs-independent oxidation, we introduce four negative control groups, in each has the absence of one component out of four key elements that support functional in vitro incubation system. Finally, with all elements on board, RM-GSH adducts are expected to be detectable and these groups work as positive controls.

With validation, we can, firstly, establish a functional incubation system of recombinant human-CYP; secondly, prove the activity of enzymes; and most importantly, demonstrate a clear pattern of CYP-dependent bioactivation.

3.2 Detection of CYP-mediated INH bioactivation

Since INH bioactivation was hypothesized in a CYPs-dependent manner, we utilize the CYP incubation systems, validated above, to investigate trapped INH-NAL adduct. To match INH bioactivation and validated substrate bioactivation side by side for pattern identification, INH

bioactivation studies also contain four control groups to rule out CYP-independent reaction. Groups with all four elements of a functional in vitro enzymatic system will be marked as the observing group.

To guarantee the reliability of these studies, we have three replicated incubation (n=3) in every group. Both validation and detection of the same individual CYP were performed in one same run together. Before each run, a blank sample, which contains only 1xPBS and incubated and prepared with the rest of the samples, was injected as the very first to detect contaminations might exist in the UPLC-MS system. All these blank samples had no signal in corresponding channels.

Here we present the representative chromatogram and MS/MS fragmentation of INH-NAL adduct in Figure 2 to confirm the structure and ensure our target channel is accurate.

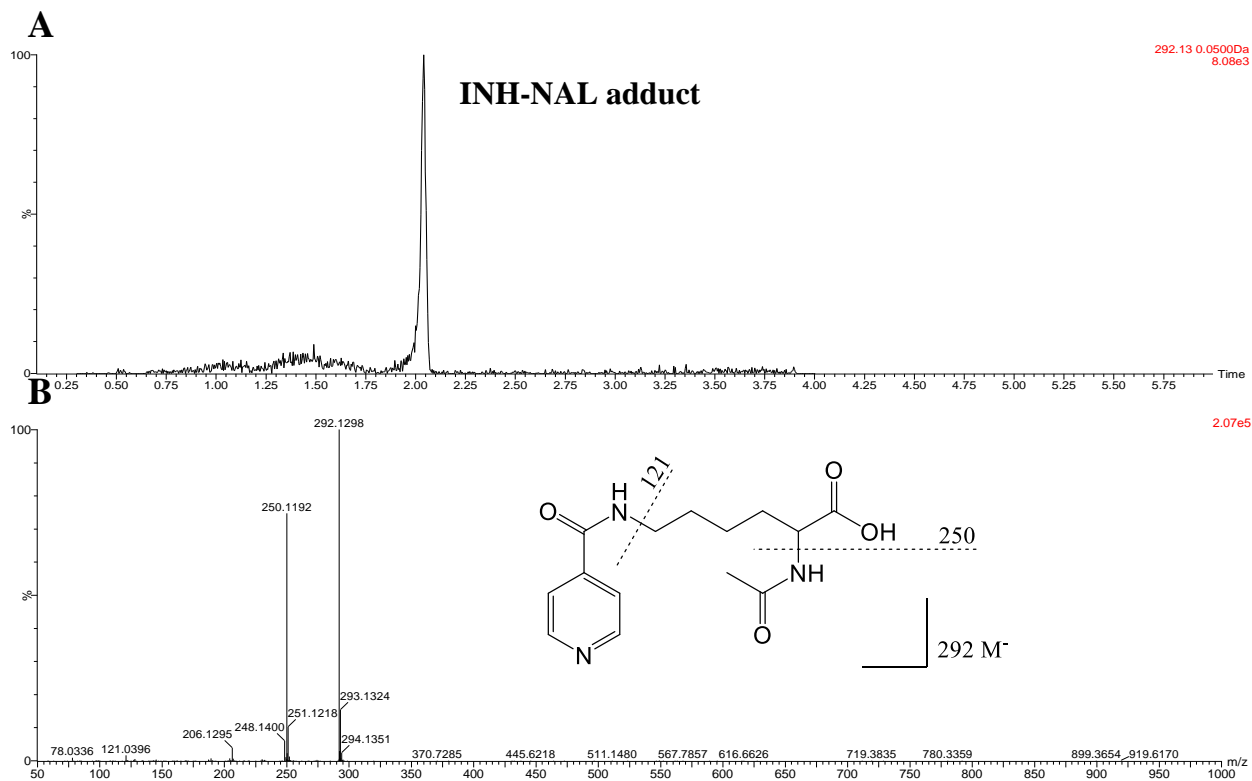


Figure 2 Trapped INH reactive metabolites

(A) Representative chromatogram of INH-NAL adduct (B) MS/MS spectrum of INH-NAL adduct.

3.3 Criteria for identification of CYP-mediated INH bioactivation

Supposedly, there will be no detection of RM-GSH signal in all four negative controls in the validation studies. Meanwhile, there will be a high response in the positive control groups, matches what had been reported previously. This contrast between the negative and positive control groups in the validation studies, the non-detection versus high response, will be considered as the pattern of a CYP-mediated substrate bioactivation.

If INH bioactivation fits into the validated individual CYP-mediated bioactivation pattern, it could be a piece of crucial evidence to support not only the existence of CYP-mediated INH bioactivation but also the supposed immune response triggered by this bioactivation-binding product, RM-CYP adducts.

In this study, we will use the pattern similarity as the criteria to identify CYP-mediated INH bioactivation.

4.0 Results

4.1 Role of CYP2E1 in INH bioactivation

4.1.1 Validation of CYP2E1 incubation system

Acetaminophen (APAP) is a well-known CYP2E1 substrate ^{63 64 53}, and its toxicity is due to this RM from CYP2E1 metabolism, particularly. APAP went through CYP2E1-mediated oxidation and formed toxic RM, NAPQI. With GSH, NAPQI was stabilized as APAP-GSH adduct. These reactions in the incubation system were demonstrated in Figure 3A. Structure identification of APAP-GSH adduct by secondary mass spectrum is demonstrated in Figure 3B.

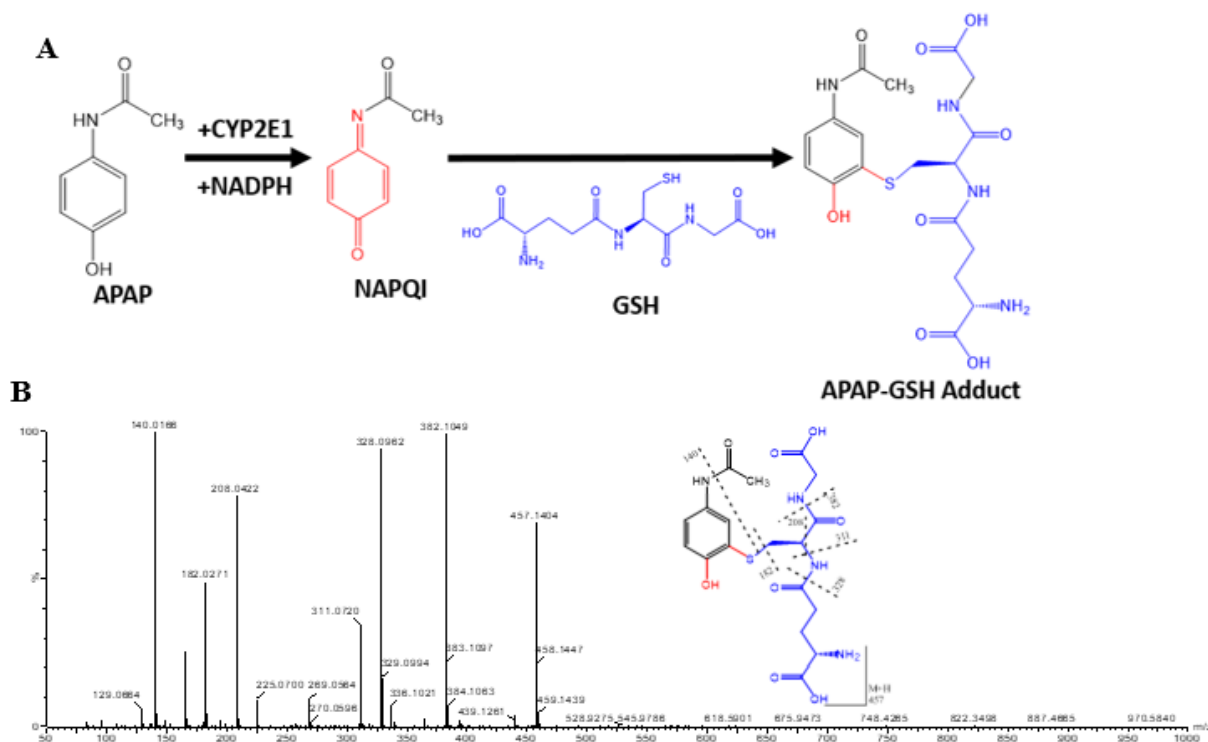


Figure 3 CYP2E1-mediated APAP bioactivation

(A) Acetaminophen can be metabolized by CYP2E1 into NAPQI; NAPQI can then get trapped by GSH and form APAP-GSH adduct. (B) MS/MS spectrum of APAP-GSH adduct.

4.1.2 Detection of CYP2E1-mediated INH bioactivation

As shown in Figure 4, four negative controls groups have barely detectable APAP-GSH adduct, while the positive control group has high-level of it. Results from this section match the previous research, suggest a functional in vitro enzymatic system and good activity of recombinant human-CYP2E1.

With its validation, we parallely demonstrated the detection of INH bioactivation via CYP2E1 with same incubation system in Figure 4A. From Figure 4B, a CYP-independent auto-oxidation was detected in the group with only INH and NAL, while the observe showed a less

abundance of INH-NAL production comparing to some of the negative control groups, such as the group without CYP/NADPH. These results suggest that the formation of INH-NAL adduct in the observe group is not mediated by CYP2E1.

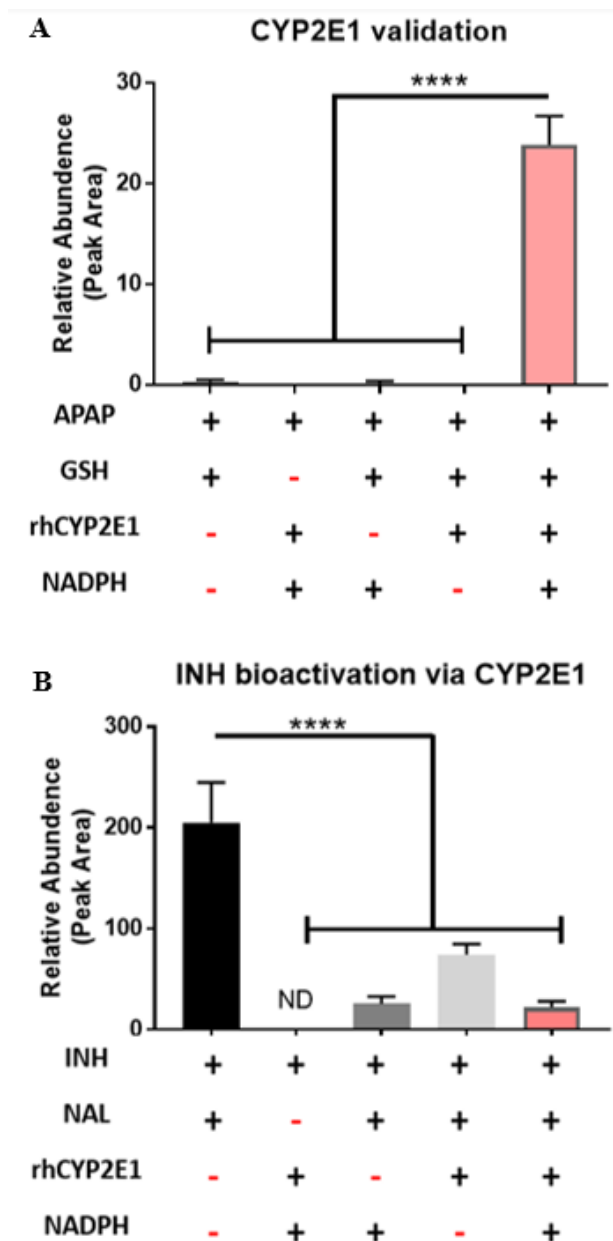


Figure 4 Role of CYP2E1 in INH bioactivation

(A) Quantitative results of APAP-GSH adduct in CYP2E1-mediated APAP bioactivation (B) Quantitative results of INH-NAL adduct in CYP2E1 and INH bioactivation. Tables underneath each graph indicate the component of each system

4.2 Role of CYP3A4 in INH bioactivation

4.2.1 Validation of CYP3A4 incubation system

Lopinavir (LPV) was previously reported as a CYP3A4 substrate.⁶⁵ It can be metabolized by CYP3A4 (predicted oxidized moiety was highlighted in red) and form RM. With GSH, RM of LPV was trapped as LPV-GSH. Reactions mentioned above in the incubation system were demonstrated in Figure 6. Structure identification of LPV-GSH by secondary mass spectrum is demonstrated in Figure 6B.

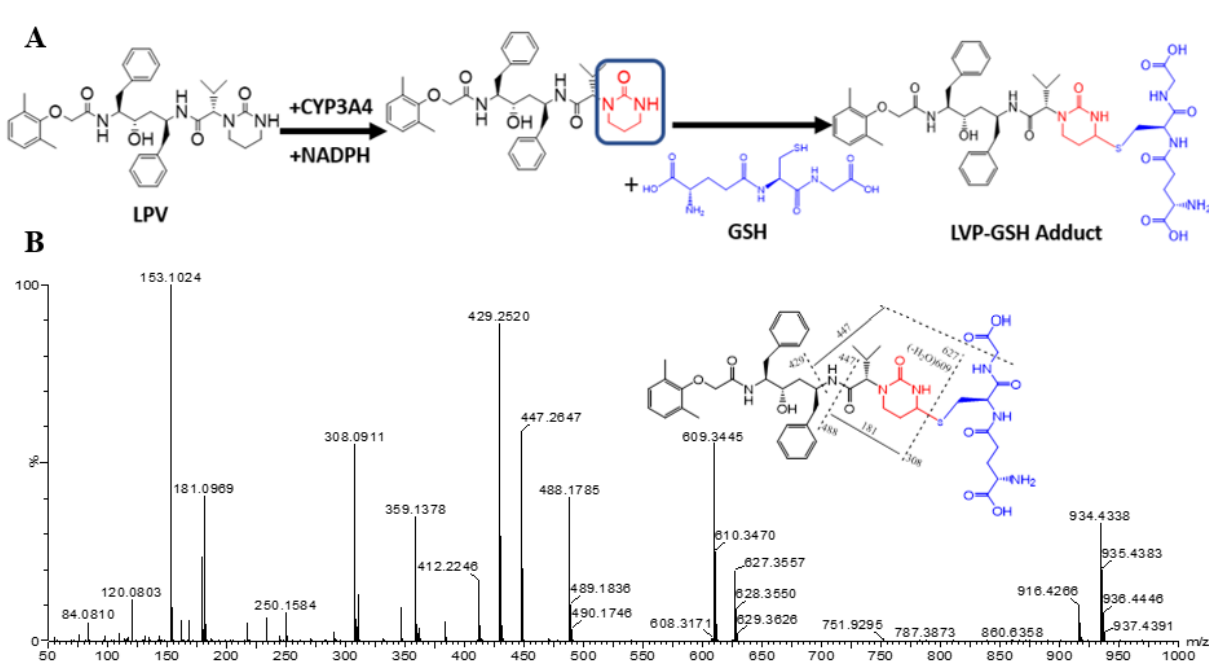


Figure 5 CYP3A4 mediated LPV bioactivation

(A) Lopinavir is metabolized by CYP3A4 and the metabolites can be trapped by GSH. (B) MS/MS spectrum of LPV-GSH adduct.

4.2.2 Detection of CYP3A4-mediated INH bioactivation

As shown in Figure 7A, four negative controls groups have an undetectable level of LPV-GSH. In its positive control group, high abundance of LPV-GSH was detected. Results here match the previous research, suggest a properly functioning in vitro enzymatic system with the fine activity of rhCYP3A4.

Alongside its validation, we parallelly demonstrated the detection of INH bioactivation via CYP3A4 with the same system. From Figure 7B, a CYP-independent auto-oxidation was detected in the group with only INH and NAL, which is the same as observed in the CYP2E1 section. Observe group showed a lower response of INH-NAL, comparing to some of the negative control groups, especially to the group with INH and NAL only. This indicates the formation of INH-NAL adduct in observe control group is not mediated by CYP3A4.

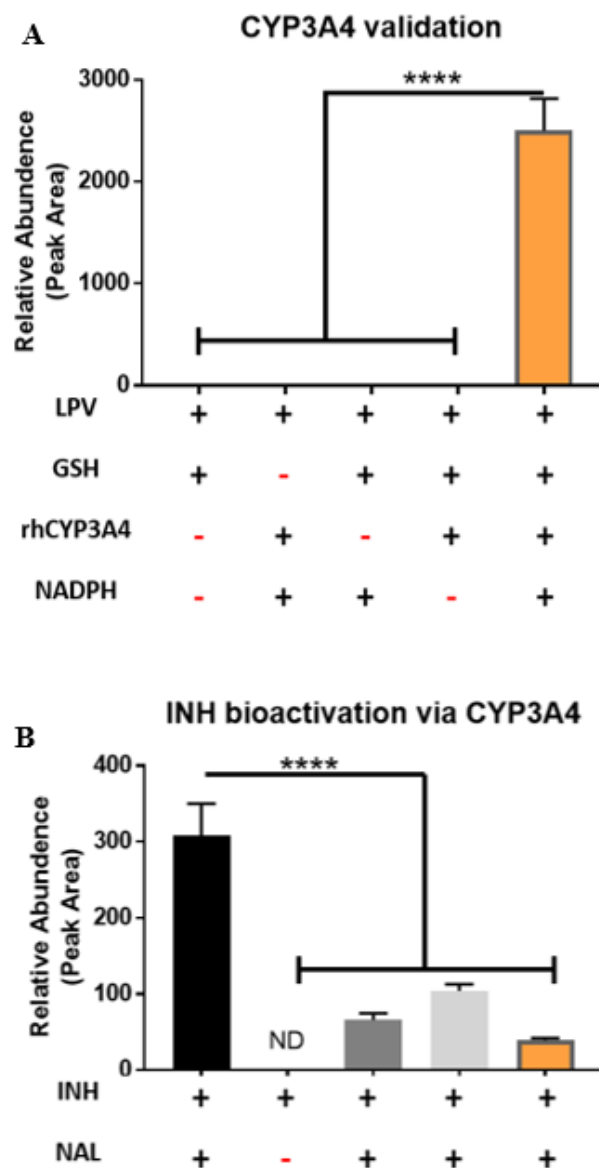


Figure 6 Role of CYP3A4 in INH bioactivation

(A) Quantitative results of LPV-GSH adduct in CYP3A4-mediated LPV bioactivation. (B) Quantitative results of INH-NAL adduct in CYP3A4 and INH bioactivation. Tables underneath each graph indicate the component of each system

4.3 Role of CYP2C9 in INH bioactivation

4.3.1 Validation of CYP2C9 incubation system

Diclofenac (DCF) is a published CYP2C9 substrate.⁶⁶ DCF went through CYP2C9-mediated bioactivation and formed its RMs (one of these structures is shown in Figure 8 and the oxidized structure was highlighted in red). With GSH, RMs of DCF was trapped as DCF-GSH adduct. The simplified reaction processes in the incubation system were shown in Figure 8A. Structure identification of DCF-GSH adduct by secondary mass spectrum is demonstrated in Figure 8B.

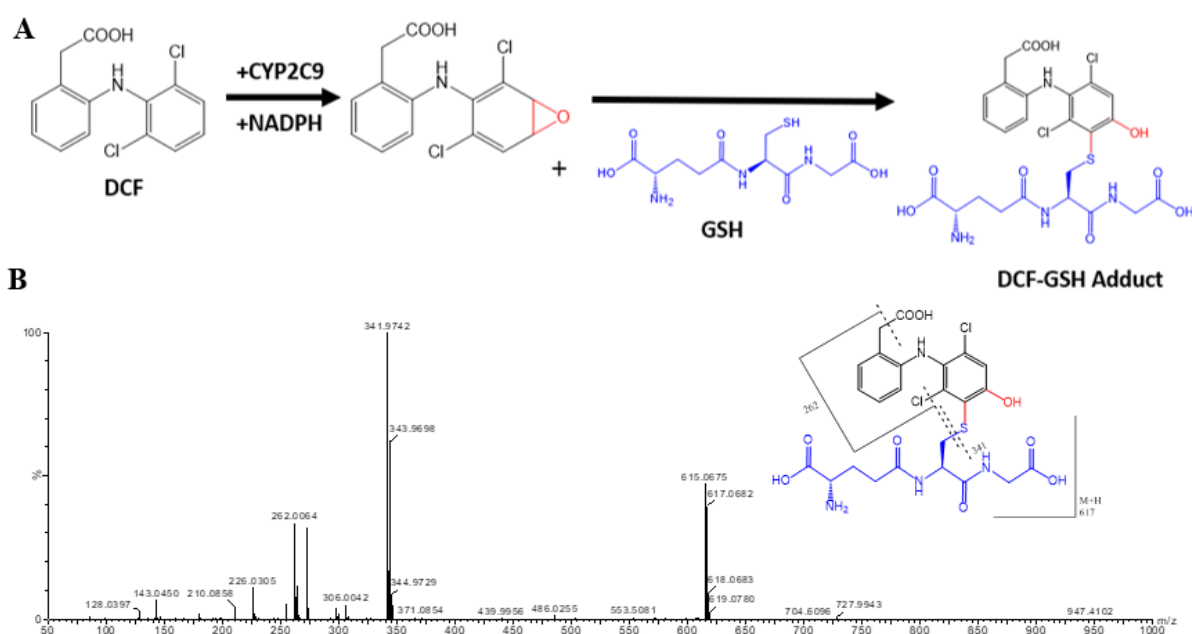


Figure 7 CYP2C9-mediated DCF bioactivation

(A) Diclofenac can be metabolized by CYP2C9 into its reactive metabolites, then can be trapped by GSH in the form of DCF-GSH adduct. (B) MS/MS spectrum of DCF-GSH adduct.

4.3.2 Detection of CYP2C9-mediated INH bioactivation

As shown in Figure 9A, four negative controls groups have very low to the undetectable level of DCF-GSH. At the same time, a high level of DCF-GSH was detected in the positive control. Results from this validation study reproduce the conclusion of previous researches, suggest a workable in vitro enzymatic system and reliable activity of recombinant human-CYP2C9.

Similarly, we demonstrated the detection of INH bioactivation via CYP2C9 with same incubation system to its validation side by side. From Figure 9B, a CYP-independent auto-oxidation was detected in the group with only INH and NAL, same as seen in two other CYPs. Observe group showed a less INH-NAL production comparing to some of the negative control groups, except the group lack of NAL. This reading supports the formation of INH-NAL adduct in the observe group is not mediated by CYP2C9.

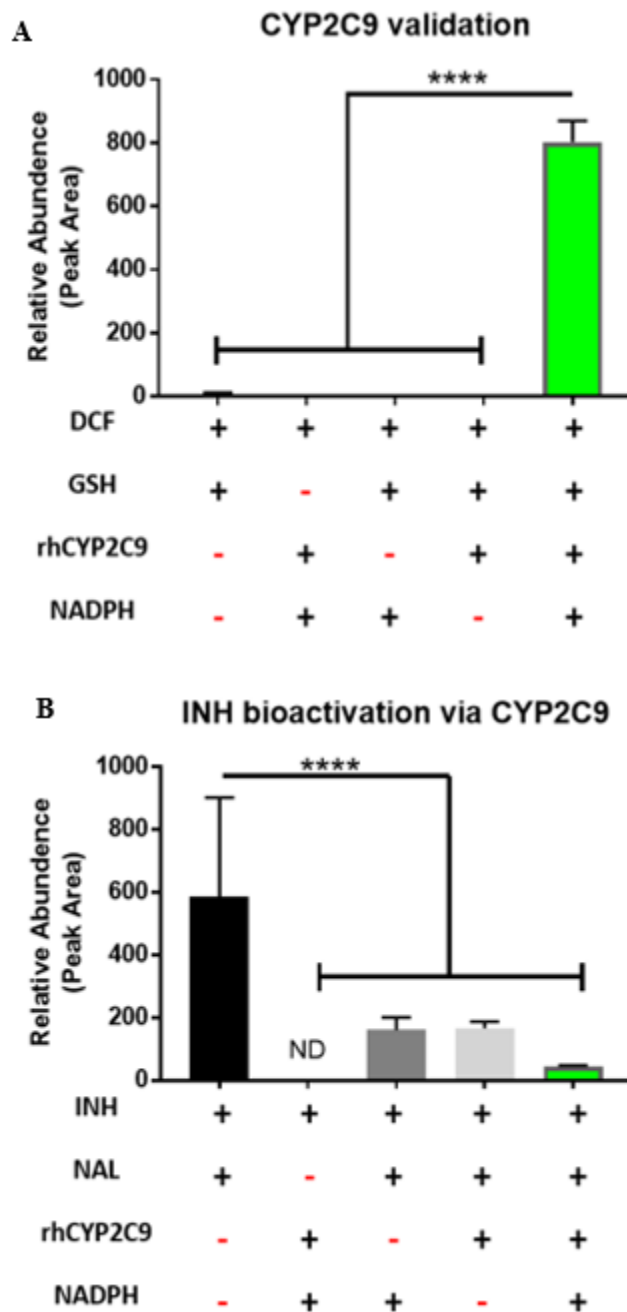


Figure 8 Role of CYP2C9 in INH bioactivation

(A) Quantitative results of DCF-GSH adduct in CYP2C9-mediated Diclofenac bioactivation. (B) Quantitative results of INH-NAL adduct in CYP2C9 and INH bioactivation. Tables underneath each graph indicate the components in each system

4.4 INH bioactivation in human liver microsome (HLM)

Evidence supports the idea of CYP mediates INH bioactivation are from the in vitro HLM incubation studies. Results from the previously published work shown that when INH and the trapping agent NAL with HLM and NADPH, reactive metabolites can be detected in the form of INH-NAL adduct. However, our results from the previous section rule out the major CYP that was hypothesized mediate INH bioactivation. Therefore, here we perform previously works of INH bioactivation via HLM and try to see if any other elements in the HLM could be the one mediates INH bioactivation.

Same as our previous system, we include four negative control groups following the same principle and one group that contains ingredients to make CYP in the HLM functional. This group is highlighted in blue. Results of HLM in INH bioactivation are shown in Figure 10.

From this study, we can see a similar result reported previously. Comparing with the group without NAL, the group that consists of every element of functional enzymatic incubation (highlighted in blue) has a high abundance of INH-NAL adduct. However, once we include other three negative control groups, we can clearly see the auto-oxidation and a similar level of INH-NAL in the group without NADPH, which further confirms the results from our studies on individual CYP. Even we can have the similar result from the published work, we can still confirm that CYP, whether in the form of recombinant individual enzymes or in HLM, are not involved in the INH bioactivation.

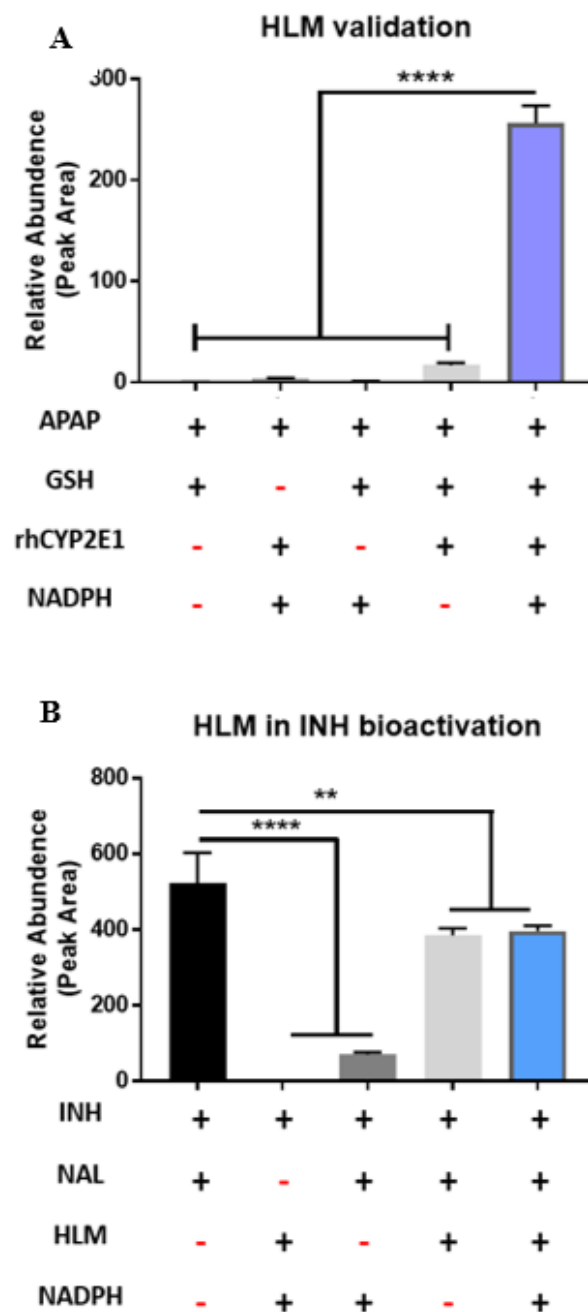


Figure 9 Role of HLM in INH bioactivation

(A) Quantitative results of APAP-GSH adduct in HLM-mediated APAP bioactivation. (B) Quantitative results of INH-NAL adduct in HLM and INH bioactivation. Tables underneath each graph indicate the components in each system.

5.0 Discussion

5.1 Method validation of CYP incubation system and CYP-mediated bioactivation

All three validation studies share pattern similarities and match the supposed contract. Thus, we have reliable systems to work with. There might be a very low response in several control groups, these are due to the noise in the channel, thus still considered acceptable. At the same time, positive controls have a clear peak in their channels.

5.2 INH bioactivation via individual CYP

CYP-independent auto-oxidation of INH, detected in the form of INH-NAL, was consistently appeared in each individual CYP sections. Moreover, this auto-oxidation has much higher efficiency comparing to CYP-mediated oxidation, due to the high abundance of INH-NAL in INH-NAL group. Although auto-oxidation would still happen in incubations with the existence of other components, we do see less INH-NAL abundance in these groups (INH-NAL-enzyme / INH-NAL-NADPH), which suggests that there is/are certain component(s), either in the recombinant human-CYP or NADPH itself, has inhibitory effects on auto-oxidation, through unknown manner.⁶⁷

After observing this auto-oxidation, we did try to eliminate it by adding in reductive substance, such as vitamin C. Somehow, we can still see the production of INH-NAL in the negative control that has neither enzyme nor NADPH. Whether the reductivity of vitamin C was

not strong enough, or there were other reasons, we could not clean up the auto-oxidation in the incubation system and have an ideal negative control as shown in the validation studies. Even if this vitamin C can work as it supposed to, the impact it will bring to the CYP-mediated oxidation is unknown. Other approaches, such as reducing oxygen in the system, might be worth trying.

Data from the vitamin C study was not shown in this thesis.

5.3 Comparison of pattern

As compared side by side, we can see similarities in each pair of validation and INH bioactivation. All probe substrates do not show any form of auto-oxidation that can be trapped by GSH, which is very different from auto-oxidation of INH. Also, each functional individual CYPs does not increase the abundance of INH bioactivation product (INH-NAL) as the probe substrates. These unmatched patterns indicate the bioactivation of INH is not mediated by individual CYPs tested above, not to mention the hypothesized immune response from adducts of bioactivated INH with these CYPs.

5.4 Conclusion

According to results from both individual rhCYP and HLM, we conclude that CYPs do not contribute to the formation of INH-NAL adduct. Immune responses stimulated by the INH-CYP adducts might not be the direct outcome of CYP-mediated INH bioactivation, and still, need more work to prove the mechanism behind.

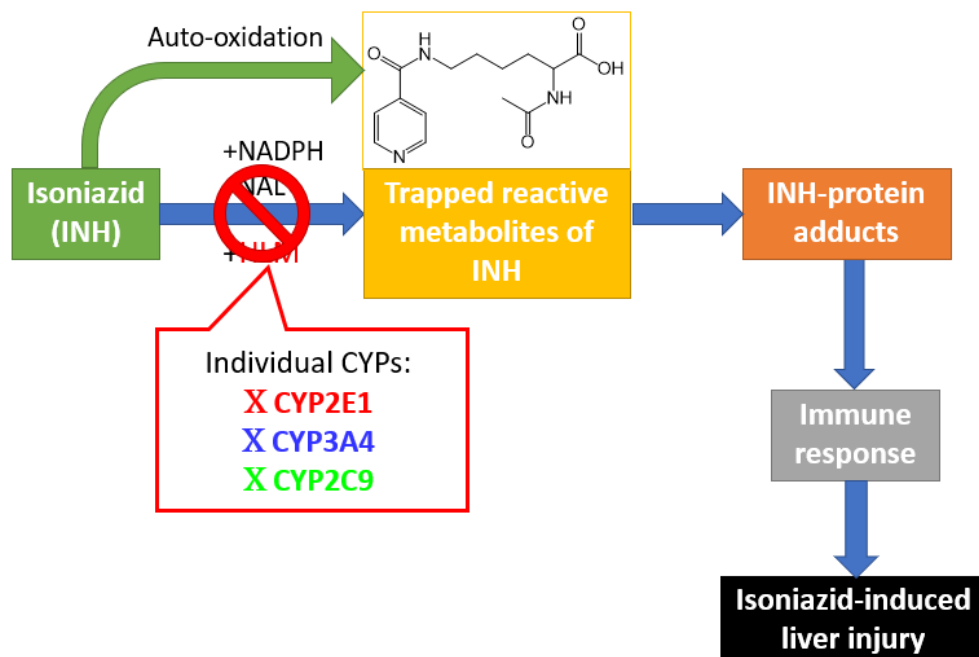


Figure 10 Study summary

No data from our study that CYP participates in the bioactivation of INH. Our data also raises concerns for the mechanism of immune responses from INH bioactivation.

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